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Xenobiotic Metabolism and Berry Flavonoid Transport across the Blood−Brain Barrier†

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A compelling body of literature suggests berry phytochemicals play beneficial roles in reversing age-related cognitive impairment and protect against neurodegenerative disorders. Anthocyanins are bioactive phytochemicals in berries suspected to be responsible for some of these neuroprotective effects. The plausible mechanisms of anthocyanin bioactivity in brain tissue are dependent on their bioavailability to the brain. Pigs were fed 2% whole freeze-dried, powdered blueberry in the diet for 8 weeks. Anthocyanin and anthocyanin glucuronides were measured in the cortex, cerebellum, and midbrain and diencephalon by LC-MS/MS. Anthocyanins and their glucuronides were found in the range of femtomoles per gram of fresh weight of tissue at 18 h postprandial, after anthocyanins had been removed from the blood by xenobiotic metabolism. Xenobiotic metabolism, anthocyanin interaction, and transporter barriers to brain bioavailability are briefly discussed. The plausible mechanism of neuroprotective action of anthocyanins may be via modulation of signal transduction processes and/or gene expression in brain tissue rather than by direct antioxidant radical quenching.

KEYWORDS: Berries; blueberries; anthocyanins; brain; bioavailability; xenobiotic; hormesis; metabolism

INTRODUCTION

Although mammals quickly remove polyphenolic compounds from the body via xenobiotic metabolism (*1*), evidence nevertheless suggests that flavonoids may protect neurological tissues by reducing neuroinflammation, to contribute to decreased age-related neurodegenerative disorders and cognitive decline (*2*). The potent antioxidant properties of flavonoids had led to the belief that their in vivo mechanism of action was via antioxidant mechanisms. However, their low bioavailability and their in vivo treatment as xenobiotics means that their direct radical quenching in vivo is negligible, especially compared to that of endogenous antioxidant defenses (*3*). However, research of the past decade has clearly demonstrated that flavonoids are potent modulators of cell signal transduction and capable of altering hundreds of metabolic pathways once present in tissues (*4*). As discussed above, the importance of flavonoids in human health may also depend on their action as hormetic phytochemicals.

Many studies of flavonoid bioavailability to circulating blood exist; however, despite numerous studies of flavonoid/drug transporter interactions (*5*), less is known about flavonoid bioavailability to brain tissue. To understand whether anthocyanins and/or anthocyanin metabolites could affect signal transduction pathways and hormetic mechanisms in the brain, it is critical that their bioavailability to the brain be ascertained.

In most cases, when plant flavonoid glycosides are consumed, they are quickly metabolized to aglycones, which are then extensively biotransformed and conjugated during absorption and hepatic metabolism (*6*). Many unanswered questions remain regarding what forms of flavonoids can be found in tissues after flavonoid feeding and what, if any, in vivo bioactivity these forms may have. A clear understanding of the relative bioavailability of individual flavonoids to the brain is further hampered by the fact that only a few compounds from each flavonoid class have been examined to date. Frequently these studies are conducted using high (pharmacological) doses; however, a few studies have used doses that are achievable in the diet using foods or extracts that have a high flavonoid concentration (*7*−*9*). Given the number and diversity of flavonoids available in diets rich in fruits, vegetables, grains, and nuts, there is considerable work yet to be done. Fortunately, analytical instruments and techniques are improving yearly and are now providing the sensitivity necessary to quantify the low levels of flavonoids found in tissues.

All interstitial tissues in the body are protected from exogenous compounds by barriers formed by the vascular endothelium, a class of epithelial cells lining the intima of blood vessels, that plays a critical role in maintaining interstitial tissue fluid homeostasis and determining which macromolecules gain passage into interstitial tissues (*10*). The brain and neurological tissues are protected by a structurally unique endothelial barrier, that is, the blood−brain barrier, which differs from other vascular barriers in its physical characteristics including the nature and number of transporters it possesses (*11*). Direct measurements reveal that flavonoid classes differ in their ability to cross the blood−brain barrier (*12*, *13*), and these differences appear to be dependent in part on the particular flavonoid's lipophilicity and polarity. As a consequence, O-methylation and glucuronidation during phase II

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metabolism may have a significant impact on flavonoid bioavailability to the brain. Little research has been conducted on the effects of flavonoid structure on bioavailability. However, research on morphine−glucuronide access to the brain (*14*) suggests there might be uptake mechanisms capable of transporting glucuronide conjugate into the brain. Evidence exists from in situ research demonstrating that P-glycoprotein transporters play a role in the flux of flavonoids into the brain (*15*). It is clear that berry anthocyanins can cross the blood−brain barrier in rodents (*16*, *17*) and can be found in the brain and ocular tissues of pigs after blueberry feeding (*18*). In this study we determine the concentrations of anthocyanins and their glucuronides in regions of the brain at a time point when anthocyanins had already been eliminated from plasma. Although the assessment of anthocyanins in tissues after their removal from plasma may likely underestimate peak concentrations attained, our goal is to assess whether sufficient anthocyanins are present in brain tissues after long-term supplementation to either act as antioxidants or induce hormetic effects.

MATERIALS AND METHODS

Chemicals. HPLC grade water was produced via a Modulab MLU filter unit (U.S. Filter Corp., Sturbridge, MA). Methanol was obtained from Fisher Scientific (Pittsburgh, PA). Formic acid and trifluoroacetic acid were obtained from Sigma Chemical (St. Louis, MO). Authentic anthocyanin standards were obtained from either Polyphenols AS (Sandes, Norway) or Extrasynthase (Genay, France). They included delphinidin-3-glucoside, cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-glucoside, peonidin-3-galactoside, peonidin-3-arabinoside, petunidin-3-glucoside, malvidin-3-glucoside, malvidin-3-galactoside, and malvidin-3,5-diglucoside.

Animals. The feeding study was conducted at the University of Prince Edward Island with the approval of the University of Prince Edward Island Animal Care Committee, under the Guidelines for the Care and Use of Experimental Animals (Canadian Council on Animal Care). Healthy neutered 32−41-day-old male pigs (Yorkshire × Landrace) were obtained from a Prince Edward Island producer at approximately 2 weeks after weaning and weighed 15 ± 2 kg at the beginning of the study (*18*). The pigs were housed in pens containing five animals each and acclimatized for 2 days on the control diet (0% blueberry) prior to being fed the experimental diets. The experimental diets were either a basal control (no blueberry powder) diet or a basal diet supplemented with 2% (w/w) whole freeze-dried, powdered blueberry (*Vaccinium corymbosum* L. cv. 'Jersey') The basal diet composition was previously defined (*18*). To balance the diet for the additional sugar present in the blueberry powder, sugars were adjusted in the blueberry-supplemented diets such that fructose and glucose were, respectively, 7 and 6 g/kg of diet. Pigs (n = 5 per BB diet group) consumed food ad libitum for 8 weeks. Animals were fasted for 18−21 h before euthanasia.

Pig Brain Sample Preparation. Blood was collected from ketamine-sedated pigs before euthanasia using pentobarbital, and brains were removed within 0.5 h after euthanasia. Brains were rinsed with water to remove excess blood. Brain regions were dissected within 0.5 h of removal from the body and flash frozen in liquid nitrogen. Brains were stored at −80 °C until freeze-drying and then returned to −80 °C storage after freeze-drying until extraction for anthocyanins. Methods for the extraction of anthocyanins have been previously described (*18*). Briefly, a portion of freeze-dried tissue was powdered, and a weighed amount [0.375 g, equivalent to approximately 1.5 g of fresh weight (FW)] was rehydrated to its original fresh tissue weight by the addition of 1−2 mL of 0.1% trifluoroacetic acid in water, vortexed for 10 s, and then sonicated for 5 min. Two milliliters of 0.4 M phosphate buffer (pH 3.9) containing 0.1% ethylenediaminetetraacetic acid was added to the samples, which were then vortexed and gently mixed for 10 min. Protein and phosphate were removed from the sample with 12.5 mL of acetone containing 0.1% trifluoroacetic acid with gentle mixing for 10 min. After centrifugation (5 min at 14000 rpm, 4 °C), the supernatant was acidified using 100 μL of neat trifluoroacetic acid. Solvent partitioning was used to remove acetone and nonpolar and polar lipids and to recover the aqueous portion of the extract. The acidified supernatant was combined with 18 mL of an organic partitioning solvent that was made by sequentially adding 6 mL each of hexane, dichloromethane, and ethyl acetate, in that order. The combined supernatant and partitioning solvent was vortexed and centrifuged, and the upper organic layer was discarded. The remaining aqueous layer was extracted with 6 mL of dichloromethane. Following vortexing and centrifugation, the top aqueous portion was removed and freeze-dried and then weighed. Dried tissue extracts were stored at −80 °C until HPLC-MS/MS analysis.

Collection of Anthocyanin Glucuronides in Human Urine. Because standards for anthocyanin glucuronides are not commercially available, these compounds were collected from the urine of a 59-year-old subject who had consumed 450 mL of a 1% (w/v) solution of a standardized bilberry (*Vaccinium myrtillus*) spray-dried powder containing 25 g of anthocyanin/100 g of powder (Artemis International, Inc., Fort Wayne, IN). Urine as collected before and at various times (30−420 min) after ingestion in a protocol similar to that of Cooke et al. (*19*). The pH of the urine samples was adjusted to pH 4.5 and divided equally into three portions. One portion was immediately chilled. A second portion was treated with glucuronidase type HP-2 from *Helix pomatia* (Sigma, St. Louis, MO) added to a concentration of 10000 U/mL of urine at 37 °C for 120 min, whereas the remaining portion was maintained at 37 °C for 120 min. Unfiltered urine from glucuronidase-treated and untreated samples (10 mL) was acidified by the addition of 40 μL of 6 mol/L hydrochloric acid immediately after enzymatic treatment. All samples were analyzed immediately, and extra sample was frozen for subsequent use. Anthocyanins and anthocyanin glucuronides were extracted from the urine samples using disposable C18 solid-phase extraction cartridges (Waters Sep-Pak Vac 12 cc C18−2 g). Cartridges were preconditioned with 7 mL of acidified methanol (0.1% trifluoroacetic acid, pH 2.1), followed by 7 mL of acidified water (10 mmol citric acid/L). Ten milliliters of the acidified urine sample was diluted with 10 mmol/L citric acid (1:1 v/v), vortexed, and loaded directly onto the solid-phase extraction cartridge. The samples were drained under gravity (∼1 drop/s) and washed with 2 volumes of acidified water (10 mmol/L citric acid, ∼12 mL). The anthocyanins were eluted from the Sep-Pac with 6 mL of acidified methanol (0.1% trifluoroacetic acid). The eluant was evaporated at ambient temperature in a SpeedVac Plus-SC110A condenser (Savant Instruments Inc., Farmingdale, NY) to 0.1−0.5 mL, and the remaining volume was dried under nitrogen at room temperature. The dry residue was dissolved in 300 μL of 5% acetonitrile in a 1% aqueous formic acid solution for HPLC-MS/MS analysis. Recovery of the internal standard (malvidin-diglucoside) was 91 ± 5%.

LC-MS Analysis. Freeze-dried tissue extracts were dissolved in 300 μL of a solution containing 5% acetonitrile (Fisher Co., Fair Lawn, NJ) in 1% aqueous formic acid (Sigma Chemical Co.). Once dissolved, the extract was drawn into a syringe to determine its volume prior to microcentrifuging at 10000 rpm for 10 min. Chromatographic separation of anthocyanins extracted from the brain tissue was conducted using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) fitted with a Phenomenex Synergi Max-RP 80A (4 μm, 150 mm × 4.60 mm i.d.), C18 analytical column. This column was protected with a Max-RP 80A (4 μm, 4 mm × 3 mm i.d.) Phenomenex guard cartridge system. Anthocyanin separation was achieved using a gradient between aqueous 4.5% formic acid (mobile phase A) to 100% acetonitrile in 4.5% formic acid (mobile phase B) over an 80 min analytical run at a flow rate of 350 μL/min at 25 °C. The gradient profile was as follows: mobile phase B, 5% at 0 min, 12% at 12 min, 24% at 40 min, 40% between 45 and 50 min, 100% from 55 to 70 min, and then 5% from 75 to 80 min. Injection volume was 100 μL. A six-port valve was used to divert the eluant to waste until just before anthocyanins began to elute from the analytical column at 14 min. The flow was then redirected into an Agilent UV G1315A diode array detector, where absorbance was monitored between 250 and 700 nm. Once through the diode array detector, the eluant was directed to an API 3000 triple-quadrupole mass spectrometer (Applied Bioscience, Foster City, CA) equipped with a TurboIon spray source. The settings were as follows: capillary voltage, 4500 V (positive mode); nebulizer gas (nitrogen), 11 arbitrary units; curtain gas (nitrogen), 6 arbitrary units; collision gas (nitrogen), 7 arbitrary units; declustering potential, 80 V; focusing potential, 400 V; entrance potential, 10 V; and collision energy, 30 eV. The drying gas (nitrogen) was heated to 400 °C and introduced at a

pressure of 30 psi. Multiple reaction monitoring was performed with Q_1 masses at the m/z of the parent anthocyanins (anthocyanidin glycosides) and Q_3 at the m/z of the resulting anthocyanidin aglycone using a cycle time of 6.1 s with a dwell time of 550 ms. Analyst 1.4.2 software from Applied Bioscience was used for data acquisition and processing.

MS/MS scans of anthocyanins extracted from tissue were compared with authentic anthocyanin standards. Tissue-derived compounds were identified on the basis of their HPLC retention time, UV absorption profile, m/z of their molecular ions, and MS/MS fragmentation pattern. Anthocyanin concentration in brain tissue extracts and standards was calculated from area counts for the anthocyanin aglycone fragment obtained by MS/MS using coefficients determined from their calibration curves and corrected for recovery on the basis of the value obtained for the internal standard (malvidin diglucoside), which was added quantitatively by displacement pipet when the lyophilized extracts were reconstituted for analysis. The relative tissue anthocyanin concentration was converted to molar quantities, corrected for dilution factors, and expressed as femtomoles per gram of fresh brain tissue.

The retention time of various phase 2 anthocyanin glucuronide conjugates was determined by observing the appearance of peaks in the chromatogram possessing a UV signal at 520 nm and the expected parent anthocyanin−glucuronide m/z values and the correct fragmentation aglycones m/z. The presence of an anthocyanin glucuronide was confirmed by monitoring its absence in glucurionidase-treated samples. All statistics were performed using Microsoft Office Excel 2003 (11.8307.8221) SP3 (Microsoft Office Professional Edition 2003, Microsoft Corp., Bellevue, WA).

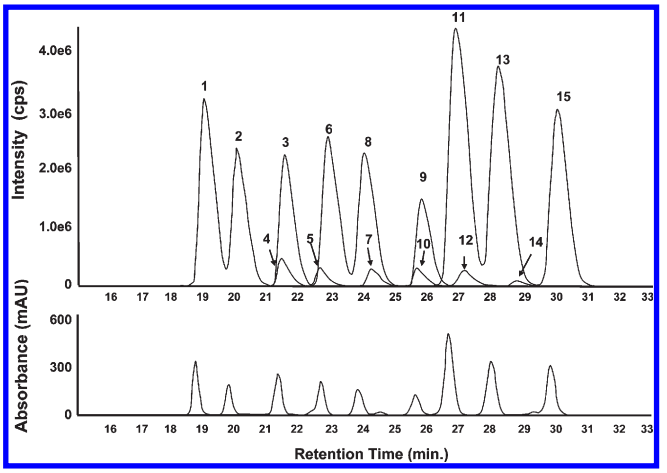

Figure 1. UV (520 nm) and aglycone m/z chromatographic trace of highbush blueberry (*V. corymbosum* L.) Jersey anthocyanin. Anthocyanins: (1) delphinidin-3-galactoside, (2) delphinidin-3-glucoside, (3) delphinidin-3-arabinoside, (4) cyanidin-3-galactoside, (5) cyanidin-3-glucoside, (6) petunidin-3-galactoside, (7) cyanidin-3-arabinoside, (8) petunidin-3-glucoside, (9) petunidin-3-arabinoside, (10) peonidin-3-galactoside, (11) malvidin-3-galactoside, (12) peonidin-3-glucoside, (13) malvidin-3-glucoside, (14) peonidin-3-arabinoside, (15) malvidin-3-arabinoside.

RESULTS AND DISCUSSION

A method has been established using HPLC-MS/MS (API 3000 triple-quadrupole mass spectrometer equipped with a TurboIon spray source) that is capable of distinguishing anthocyanins of the same molecular weight generating different aglycone fragments. Fifteen anthocyanins of Jersey blueberry (*V. corymbosum* L.) were easily identified in an acidic methanolic extract of anthocyanins from animal brain tissues after blueberry ingestion despite overlap in retention times of some anthocyanins as shown in **Figure 1**. The more prevalent anthocyanins in Jersey blueberries were malvidin-3-glucoside, malvidin-3-galactoside, malvidin-3-arabinoside, delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-galactoside, petunidin-3-glucoside, and petunidin-3-arabinoside. Present in lesser quantities in Jersey blueberries were cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, and peonidin-3-arabinoside.

Ten of the 15 Jersey anthocyanins were present in a sufficient quantity in the brain regions of pigs supplemented with 2% whole freeze-dried powdered blueberry in the diet for 8 weeks to permit quantification using authentic standards and a malvidin-diglucoside internal standard. Samples were collected from pigs that had been fasted for a period of at least 18 h and, as expected from prior pharmacokinetics studies in animals and in humans (*20*−*22*), both anthocyanins and their metabolites were below detection limits in plasma.

As previously observed, anthocyanins appear to have a longer residence time in tissues than in plasma (*18*). Anthocyanins were detected in the brains of both supplemented and unsupplemented pigs, and they were detected at higher levels in the supplemented animals. The origin of the anthocyanins in the animals fed the 0% blueberry supplemented diet was undoubtedly the control diet, so the control diet was analyzed for anthocyanins. The diets fed to pigs at weaning and the 0% blueberry basal diets were both found to contain low levels of anthocyanins that ranged from 130 to 165 ng of cyanidin glucoside equiv/g of feed, respectively. These values are equivalent to supplementation with 0.0002% blueberries in the diet and are thought to originate from anthocyanins found ubiquitously in oats, barley, and soy, which account for 70% of the basal diet. Nevertheless, supplementation with 2% blueberries in the diet showed a significant increase in the majority of blueberry anthocyanins in the cerebellum and midbrain/diencephalon regions of the brain (**Figure 2**).

In this previous study of dose and tissue distribution, anthocyanin profiles varied among the different tissues, suggesting that differential transport, metabolism, and retention may have contributed to the distinctive tissue anthocyanin profiles. In this study the relative concentrations of the anthocyanins observed in different regions of the brain were similar (**Figure 2**). All three regions of the brain (cortex, cerebellum, and midbrain and diencephalon combined) contained malvidin-3-glucoside in highest concentration, and in these pigs a mean of 279 fmol/g of tissue in the cortex to 432 fmol/g of tissue in the midbrain and diencephalon was observed at the 18 h postprandial time. It should be noted that malvidin is the most highly methylated among the six common anthocyanidin structures. It is possible that tissue malvidin-3-glucoside may include both diet-derived malvidin-3-glucoside and malvidin-3-glucoside produced by the action of catechol-*O*-methyltransferase and other methyltransferases. Other flavonoids such as quercetin are extensively methylated following ingestion by humans and animals (*23*, *24*). Catechol-*O*-methyltransferase has been shown to produce malvidin-3-glucose using petunidin-3-glucose as a substrate (*25*), and this is likely to occur in the liver. Although species differences exist between catechol-*O*-methyltransferase enzymes, the brain and liver catechol-*O*-methyltransferases within a species are identical (*26*). It is therefore possible that malvidin-3-glucoside may be generated locally within the brain from petunidin-3-glucoside and that observed levels include both absorbed and metabolized anthocyanins.

Malvidin-3-galactoside, peonidin-3-glucoside, and petunidin-3-glucoside were the next most prevalent anthocyanins in all three brain regions after consumption of Jersey blueberries. The fact that peonidin-3-glucoside was relatively abundant in the brain while not so in the Jersey blueberries suggests again that methylation of anthocyanins may be occurring. Peonidin-3-glucoside may be formed in, or translocated to, the brain where it can accumulate. Petunidin-3-glucoside would be a product of

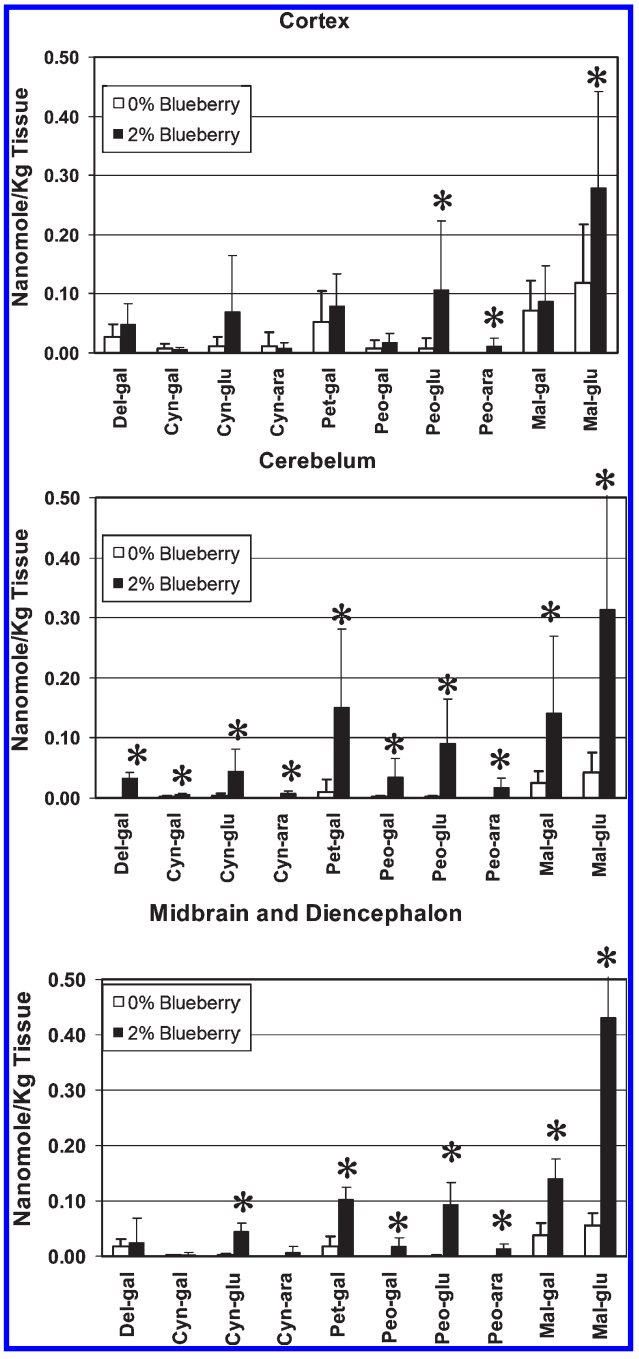

Figure 2. Tissue distribution of anthocyanins in (**A**) cortex, (**B**) cerebellum, and (**C**) midbrain and diencephalon. Open bars represent the unsupplemented (0% blueberry) diet, and solid bars represent the 2% whole freeze-dried, powdered blueberry supplemented diet. Asterisks signify significance at $p \leq 0.005$ for differences in tissue anthocyanin concentration with and without blueberry supplementation.

delphinidin-3-glucoside methylation, whereas peonidin-3-glucoside would be a methylation product of cyanidin-3-glucoside.

Andres-Lacueva et al. (*7*) suggest the presence of several anthocyanins (cyanidin-3-*O*-β-galactoside, cyanidin-3-*O*-β-glucoside, cyanidin-3-*O*-β-arabinose, malvidin-3-*O*-β-galactoside, malvidin-3-*O*-β-glucoside, malvidin-3-*O*-β-arabinose, peonidin-3-*O*-β-arabinose, and delphinidin-3-*O*-β-galactoside) in the cerebellum, cortex, hippocampus, or striatum of rats fed a 2% blueberry supplemented diet extract for 8−10 weeks. However, these investigators did not quantify the anthocyanins.

In another study in which red grape extract containing 3.8 μmol of anthocyanins (2 mg) was introduced into the stomach of rats, unmetabolized anthocyanins were observed in plasma (176 ng/mL) and in the brain (192 ng/g) within 10 min after gavaging (*27*). The whole brain was quickly and carefully rinsed in ice-cold phosphate-buffered (pH 7.4) saline solution to remove traces of meningeal blood. It is possible that these high levels may be due to some contamination by blood as rinsing the brain may not have been sufficient to remove all blood from brain tissue.

Talavera et al (*16*) fed rats with a blackberry (*Rubus fruticosus* L.) anthocyanin-enriched diet for 15 days and identified and quantified anthocyanins and metabolites. These investigators studied anthocyanin distribution to the digestive system (stomach, jejunum, liver), an excretion organ (kidney), and the brain using HPLC-ESI-MS-MS and HPLC−diode array detector. They found only native blackberry anthocyanins (cyanidin 3-*O*-glucoside and cyanidin 3-*O*-pentose) in the stomach; however, other organs (jejunum, liver, kidney) contained native, methylated, and conjugated anthocyanidins including cyanidin and peonidin monoglucuronides. In the blackberry feeding study rats were sacrificed at 3 h after the beginning of the last meal. Brain tissue was harvested from animals that had been exsanguinated and perfused with 20 mL of phosphate-buffered saline. Anthocyanins were found in the brain, and cyanidin 3-glucoside content was higher in the brain (0.21 $\pm$ 0.05 nmol/g of tissue) than in plasma (0.15 $\pm$ 0.02 nmol/mL). The authors indicate that the presence of anthocyanins in the brain was not due only to residual anthocyanins in the vessels and/or the capillary endothelium. Cyanidin 3-glucoside was the predominant form (84%) of anthocyanins observed in the brain after consumption of blackberries. Because peonidin-3-glucoside was not in an extract of blackberry but was found in urine, plasma, kidney, liver, and brain, the authors conclude that native blackberry anthocyanins and their methylated forms reached the brain. This may be true, but this conclusion ignores the possibility that anthocyanin methylation can occur within the brain.

Borges et al. (*8*) fed rats raspberry (*Rubus idaeus* var. Glen Ample) juice, and tissues were collected at various times up to 24 h after administration of juice. Liver, kidney, and brain were perfused in situ with chilled 0.15 M KCl and prior to removal with other organs including stomach, duodenum/jejunum, ileum, cecum, and colon, with their contents intact, at each time point. These investigators used HPLC-PDA-MS/MS analysis to identify and quantify nine anthocyanins, two ellagitannins, and ellagic acid. Anthocyanins were not detected in the brain of rats following the ingestion of the 2.77 mL of raspberry juice containing 918 nmol of anthocyanins.

It is possible that the differences in anthocyanin detection capability in brain tissues may be influenced to some degree by the extraction techniques, which differ significantly between the studies. Woodward et al. has investigated anthocyanin stability under simulated (in vitro) physiological conditions as well as the degradation and recovery of anthocyanins following routine preanalytical sample extraction and storage (*28*). The study showed that anthocyanins are stable when stored at −80 °C and pH ≤2, whereas anthocyanidins are relatively less stable. These investigators also suggest that poor recovery of anthocyanins following feeding studies is not the result of sample degradation during storage but rather the result of degradation in vivo or during initial sample processing and that the degree of B-ring hydroxylation mediated the degradation of anthocyanins to their phenolic acid and aldehyde constituents (*28*). We had previously found in anthocyanin stability studies in long-term-stored frozen tissue and frozen tissue extract samples that our best recovery of spiked anthocyanins was from samples that remained frozen at

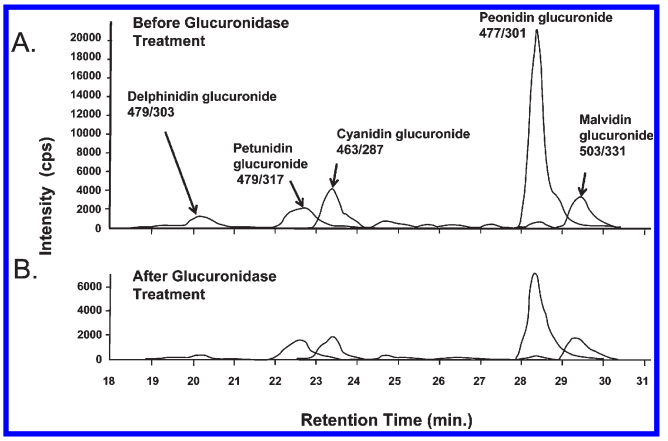

Figure 3. LC-MS/MS trace of the abundance of the aglycone fragments of the molecular ions of anthocyanin glucuronides from human urine at 120 min after consuming 1.62 g of bilberry extract: (**A**) anthocyanidin traces of untreated urine; (**B**) urine treated with glucuronidase treatment. These are the MS/MS product ions of multiple reaction monitoring. The levels of glucuronides are diminished to less than half the original values during the 2 h glucuronidase treatment.

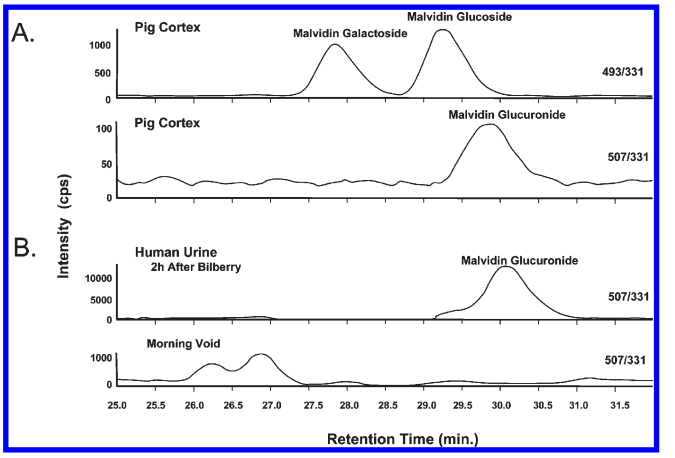

Figure 4. (**A**) LC-MS/MS trace of the aglycones of malvidin and malvidin-glucuronide from 18 h postprandial samples of pig cortex from pigs supplemented with 2% whole freeze-dried blueberry powder diet for 8 weeks. Glucuronide levels are $^{1}/_{10}$th those of the parent glycosides. (**B**) LC-MS/MS trace of the aglycones of malvidin-glycosides and malvidin-glucuronide in human urine 1 h after feeding 1.62 g of bilberry powdered extract.

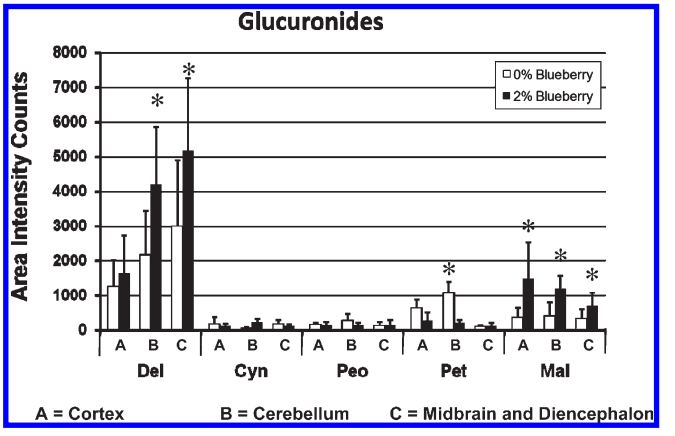

Figure 5. LC-MS/MS trace of the aglycones of anthocyanin-glucuronide from regions of the brain indicated as (A) cortex, (B) cerebellum, and (C) midbrain and diencephalon. Between 18 and 20 h after the last feeding, brain tissue was collected from pigs that had been fed a 2% whole freeze-dried blueberry powder for 8 weeks.

−80 °C until extraction. Freeze-drying samples requires that they be rehydrated prior to extraction and could result in anthocyanin losses as suggested by Woodward et al. (*28*). In our experience these losses can be minimized by bringing the samples to pH ≤3.9 as soon as possible; however, this was secondary to keeping the anthocyanins in the presence of proteins for as long as possible prior to analysis.

He et al. (*29*) and Wu et al. (*30*) investigated the fate of anthocyanins in the gastrointestinal (GI) tract in rats and pigs, respectively. Both studies found that anthocyanins were surprisingly stable in the GI tract with recoveries from pig GI contents of 41.7% of total administered anthocyanins at 4 h and 75−79% of administered dose from 30 to 120 min after delivery from rat's gastric and small intestinal contents. Nevertheless, urinary recoveries remained very low in these studies. These investigators speculate that anthocyanin degradation occurs in the GI tract via hydrolysis of glycosidic linkages occurring in the acidic gastric lumen and via selective hydrolysis by endogenous β-glucosidase activity in the small intestine and that the aglycones and associated glycosides play a role in determining anthocyanin stability. None of these studies investigated the stability of anthocyanins extracted from tissue.

A major metabolic fate of anthocyanins in vivo is via glucuronidation. Currently there are no commercially available anthocyanidin glucuronide standards. We therefore undertook to validate and characterize the chromatographic properties of the various glucuronides of *Vaccinium* anthocyanins by isolating urinary glucuronides from a human subject who had consumed bilberry anthocyanins. **Figure 3A** depicts the chromatographic peaks in a urine extract 120 min after consumption of 1.62 g of bilberry extract. These peaks exhibit the expected m/z of anthocyanin glucuronides and a corresponding aglycone fragment using multiple reaction monitoring. LC-MS/MS. **Figure 3B** illustrates the loss in the peaks of the same sample after treatment with glucuronidase type HP-2 from *H. pomatia* at 10000U/mL of urine at 37 °C for 120 min.

Figure 4A depicts a representative chromatogram of pig cortex monitored for malvidin-3-glycosides and malvidin glucuronide. This is a typical representation of all the anthocyanins where the levels of the glucuronides in brain tissue were $\sim^{1}/_{10}$th the levels observed for the parent anthocyanin glycosides. The apparent malvidin glucuronide peak is found at the same retention time and exhibits the same m/z and aglycone fragment as that appearing in human urine at 120 min after the consumption of bilberry anthocyanin.

Similar profiles are observed for the various glucuronides of Jersey blueberry anthocyanins in the three brain regions studied (cortex, cerebellum, and midbrain and diencephalon; **Figure 5**). The prevalent glucuronide was a conjugate of delphinidin. Because the glucuronides isolated from human urine were not purified, we were able to quantify the glucuronides only by their intensity relative to each other. In the cortex, malvidin glucuronide and delphinidin glucuronide were equivalent. In the cerebellum and the midbrain and diencephalon, the delphinidin glucuronide to malvidin glucuronide ratio was increased relative to the cortex to ratios of 3.5 and 7.4, respectively. Compared to the glucuronides of cyanidin, peonidin, and petunidin, the glucuronides of malvidin and delphinidin were present at levels between 6 and 48 times greater across the brain regions. At 18 h postprandial, the prevalence of delphinidin glucuronides in brain regions suggests that a large proportion of native delphinidin glycosides may become glucuronidated or that it is removed less efficiently from the brain than other anthocyanin glucuronides. These issues in determining the bioavailability and

metabolic fate of anthocyanins may not be completely resolved until radiolabeled or stable isotopically labeled anthocyanins become available.

This study has demonstrated that Jersey blueberry anthocyanins when fed to pigs are bioavailable, transit the blood−brain barrier, and can be found in brain tissue 18 h after the last anthocyanin feeding. Whether or not methylated and glucuronidated anthocyanin conjugates were synthesized in the brain or in the liver and kidney and then transported to the brain was not determined. Glucuronides of anthocyanins were identified in the cortex, cerebellum, and midbrain and diencephalon of blueberry-fed pigs. The qualitative patterns of anthocyanins and their possible phase II metabolites suggest that the delphinidins may have been preferentially glucuronidated, whereas cyanidins were methylated to peonidins. The prevalence of malvidins in the brain regions of blueberry-supplemented pigs suggests that methylation may convert delphinidin to petunidin and thence to malvidin. Equally possible is that malvidin aglycones and their glucuronides are inherently more stable and therefore reside longer than other anthocyanins in the brain. Viniculture studies have demonstrated the formation of anthocyanin−protein cross-links in red wines (*31*). It is also known that polyphenols have an affinity for protein and DNA (*32*) and that complex formation is favored in neutral and basic pH conditions (*33*). Many flavonoids including cyanindin chloride have been demonstrated to bind to lipoproteins (*34*), and the aglycones delphinidin, quercetin, and kaempferol were shown to bind to DNA via both intercalation and external binding (*35*).

The number and configuration of hydroxyl groups on the B-ring may affect the ability of polyphenolic compounds to complex with protein. Delphinidin polymers bind proteins more tightly than cyanidin polymers (*36*). The extent of this occurrence in vivo is not known, nor is it known whether binding of monomer anthocyanins is comparable to that of polymer anthocyanins; however, the notion of tight binding of anthocyanin to proteins implies that tissue extraction methods could have a significant impact on levels of anthocyanins recovered. Furthermore, the values reported here for the 18 h postprandial point are well beyond the time of expected plasma C_{max}, and lower levels of anthocyanin would thus be available for phase II metabolism within the brain. Therefore, the values reported here may underestimate the long-term mean anthocyanin concentration within tissues. Nevertheless, levels of plasma anthocyanins are low compared to other flavonoids. The research presented here suggests that anthocyanin levels in tissues are even lower and that xenobiotic metabolism continues to act on their removal from tissues and from the body even after 18 h. Considering their tissue concentration relative to endogenous glutathione, it is extremely unlikely that the primary mode of health beneficial action of anthocyanins is via direct radical quenching. Given their tissue concentrations, it is however possible that their effect on signal transduction pathways, gene expression, and other known and unknown mechanisms is responsible for their observed health effects.

Aura et al. (*37*) and Keppler and Humpf (*38*) have investigated the ability of gut microflora to degrade anthocyanins to simple phenolic acids and concluded that this is the predominant metabolic fate of consumed anthocyanins. These phenolic acids may have bioactivity and can influence and are influenced by xenobiotic metabolism and can account for some of the bioactivity derived from the consumption of anthocyanins. This study concentrated on determining if anthocyanins reach the brain and, if so, in what quantities. This information is critical to determining plausible roles of berry anthocyanins in eliciting hormesis and in providing neuroprotection.

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